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A NEW METHOD FOR DEGRADATION OF THE PROTEIN PART OF GLYCOPROTEINS: ISOLATION OF THE CARBOHYDRATE CHAINS OF ASIALOFETUIN

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ABSTRACT

A new method has been developed for degrading the protein part of several glycoproteins, whilst leaving the carbohydrate portion virtually intact apart from partial degradation at the reducing end. The method is based upon stabilization of the glycosidic linkages of the sugar residues by trifluoroacetyl groups and subsequent cleavage of the peptide bonds by transamidation. The two reactions are carried out in a mixture of trifluoroacetic anhydride and trifluoroacetic acid. After *O*- and *N*-detrifluoroacetylation, the carbohydrate portion can be isolated and re-*N*-acetylated. The applicability of the method is demonstrated by the isolation from asialofetuin of the carbohydrate chains that are attached by *N*- and *O*-glycosyl links.

INTRODUCTION

The carbohydrate chains of glycoproteins are attached to the protein part either by *N*-glycosyl links to asparagine or by *O*-glycosyl links to serine, threonine, hydroxylysine, or hydroxyproline[1]. The carbohydrate chains can be isolated as glycopeptides by extensive action of proteolytic enzymes, but often the peptide portion left is of considerable size. Carbohydrate chains attached by *N*-glycosyl links can be cleaved by hydrazinolysis[2] or by alkaline borohydride treatment[3], and carbohydrate chains attached by *O*-glycosyl links to serine or threonine can be cleaved by mild treatment[4] with alkaline borohydride.

We now report a new method by which the protein part of glycoproteins can be degraded leaving the carbohydrate portion virtually unaffected apart from partial degradation at the reducing end.

RESULTS

When asialofetuin was treated with 50:1 trifluoroacetic anhydride/trifluoroacetic acid (TFAA/TFA) at 100°, it dissolved within 3 h, giving a dark, clear solution. After 48 h, the reaction was stopped and the reagents were removed by evaporation, leaving a residue consisting of *O*- and *N*-trifluoroacetylated carbohydrates and degradation products. After *O*-detrifluoroacetylation by treatment with methanol and 50% aqueous acetic acid, the *N*-trifluoroacetylated product was freed from degradation products by extraction with ethyl ether. The resulting product was then fractionated on a column of Sephadex G-25 (Fig. 1).

The two fractions were then *N*-detrifluoroacetylated by reduction with sodium borodeuteride, acetylated, and *O*-deacetylated. Each fraction was then purified by gel filtration, to yield the fractions IR and IIR; the yields and analytical data for

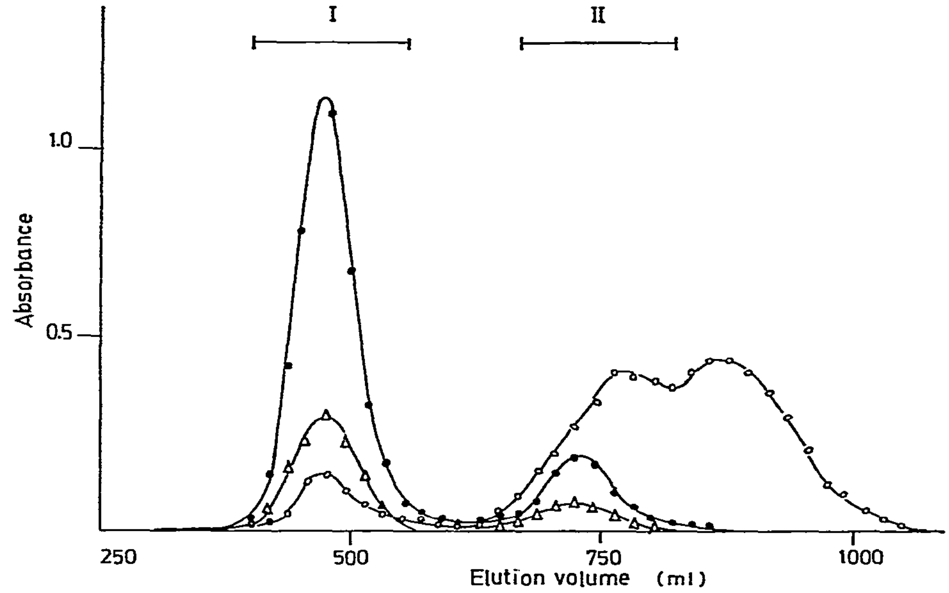


Fig. 1. Fractionation of asialofetuin on a column (3 × 140 cm) of Sephadex G-25 after reaction with TFAA/TFA and *O*-detrifluoroacetylation. Eluant: 0.1M ammonium acetate buffer (pH 7.0). The flow rate was 0.5 ml/min, and fractions (7.6 ml) were assayed for total hexose (●—●), hexosamine (△—△), and material absorbing at 280 nm (○—○). Fractions were combined as indicated.

TABLE I

YIELDS AND ANALYTICAL DATA FOR RECONSTITUTED FRACTIONS OBTAINED BY TRIFLUOROACETOLYSIS OF FETUIN

Fraction	*Yield (mg)*	*Composition (%)*				
		D-*Gal*	D-*Man*	D-*GlcNAc*	D-*GalNAc-ol*-1-d	D-*GalNAc*
IR	150.3	26.5 (3.2)[a]	24.8[b] (3.0)	41.5[c] (4.1)	—	—
IIR	50.0	22.0[d] (1.4)	—	—	18.5 (1.0)	—
Asialofetuin	1 000	4.5 (4.1)	3.3 (3.0)	7.1 (5.3)	—	1.1 (0.8)

[a] Molar ratios in parentheses. [b] This value also includes D-Man-ol-*1-d*. [c] This value also includes D-GlcNAc-ol-*1-d*. [d] Of this, 4% represents free D-Gal-ol-*1-d*.

TABLE II

METHYL ETHERS OBTAINED IN HYDROLYSATE OF REDUCED, *N*-ACETYLATED, AND METHYLATED FRACTION IR AND ASIALOFETUIN

Sugars	T[a]	*Molar ratios*	
		Fraction IR	*Asialofetuin*
2,3,4,6-*O*-Me-D-Gal	1.07	2.9	3.0
3,4,6-*O*-Me-D-Man	1.45	1.3	1.1
3,6-*O*-Me-D-Man	1.97	1.0	1.0
2,4-*O*-Me-D-Man	2.40	0.6	1.0
3,6-*O*-Me-D-GlcN(Me)Ac	4.15	+[b]	5.3
3,6-*O*-Me-D-GlcNAc	4.32		
1,2,4,5-*O*-Me-D-Man-ol-*1-d*	1.00	0.4	—[c]
1,3,5,6-*O*-Me-D-GlcN(Me)-Ac-ol-*1-d*	1.95	+	—
1,3,5,6-*O*-Me-D-GlcNAc-ol-*1-d*	2.95		

[a] Retention times (SE-30) of the corresponding alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b] Present, as determined qualitatively. [c] Absent, as determined qualitatively.

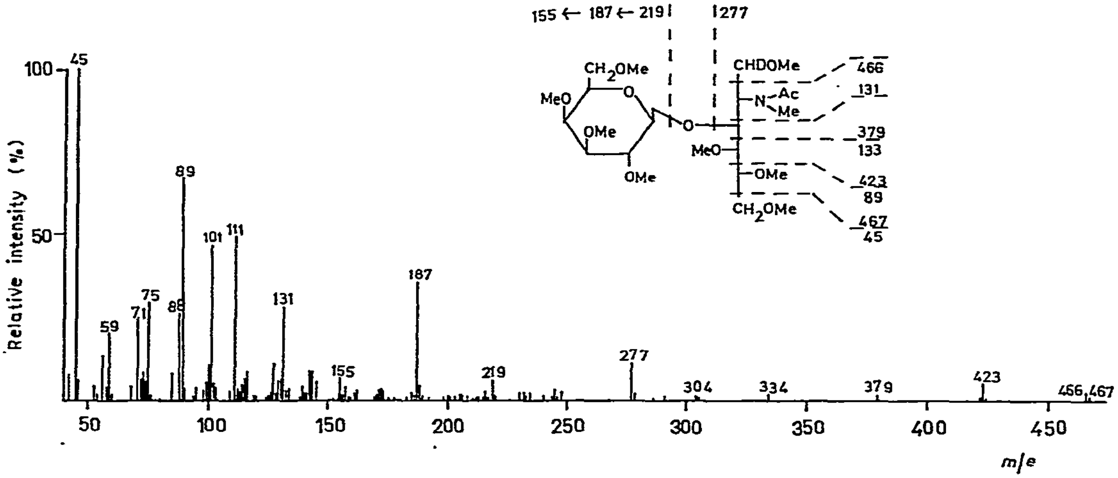


Fig. 2. Mass spectrum of the permethylated disaccharide alditol obtained from permethylation of fraction IIR, with characteristic fragmentation for a permethylated Hex*p*-(1→3)-HexNAc-ol-*1-d*.

these fractions are given in Table I. Fraction IR was further characterized by methylation analysis (Table II). Acetylation of Fraction IIR gave 4% of D-galactitol-*1-d* hexa-acetate. Fraction IIR was also permethylated, and analysis by g.l.c.–m.s. revealed a single component in the disaccharide region; the mass spectrum (Fig. 2) shows fragments typical[5] for a permethylated Hex*p*-(1→3)-HexNAc-ol-*1-d*.

DISCUSSION

We have shown[6] that glycosidically linked sugars are stable to the action of

50:1 TFAA/TFA at 100° for 48 h. This stability of the glycosidic bond is due to the inductive effect of the *O*-trifluoroacetyl groups of the pertrifluoroacetylated sugars rapidly formed in the reagent mixture. The greatest stabilizing effect is given by the 2-*O*-trifluoroacetyl group in the glycon and the *O*-trifluoroacetyl groups adjacent to the glycosidic linkage in the aglycon sugar residue. Analogous results have been obtained in acetolysis experiments[7]. However, the TFAA/TFA reagent transamidates the *N*-acetyl group of 2-acetamido-2-deoxy sugars[8], and thus also peptide linkages, to form *N*-trifluoroacetyl derivatives within 48 h at 100°.

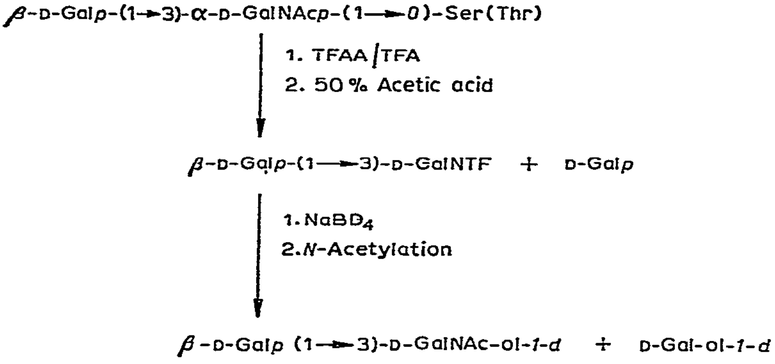
We have now investigated the possibility of degrading the protein part of glycoproteins and recovering the carbohydrate chains intact and free from protein. Since the structures of the carbohydrate chains of fetuin have been extensively studied[9-12], asialofetuin was chosen as a model. Asialofetuin was degraded with 50:1 TFAA/TFA, to yield an *O*- and *N*-trifluoroacetylated product. After *O*-detrifluoroacetylation, the *N*-trifluoroacetylated carbohydrates were recovered, and fractionated by gel filtration (Fig. 1). In order to reconstitute the original *N*-acetyl groups of the 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose residues, the *N*-trifluoroacetyl groups were removed by reduction with sodium borodeuteride[8]. The reduction step also converts a reducing sugar unit into an alditol residue labelled with a deuterium atom at C-1. *N*-Acetylation was carried out in two steps, *i.e.*, *O*- and *N*-acetylation followed by *O*-deacetylation.

The treatment with 50:1 TFAA/TFA results in degradation of the protein and elimination (by cleavage of the 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-D-glycopyranosylaminic bond) of the carbohydrate chains attached by *N*-glycosyl links. The initially formed, pertrifluoroacetylated, 4-substituted 2-deoxy-2-trifluoroacetamido-1-*N*-trifluoroacetyl-β-D-glucopyranosylamine residue at the reducing end is then further partially degraded by acid-catalyzed elimination, generating a pertrifluoroacetylated, 4-substituted 2-deoxy-2-trifluoroacetamido-D-glucose residue at the reducing end. This residue is, in turn, partly degraded[13], exposing the stable 3,6-disubstituted D-mannose residue (Scheme 1). The product was *O*-detrifluoroacetylated by treatment with methanol and 50% aqueous acetic acid, and *N*-detrifluoroacetylated by reduction with sodium borodeuteride. The reduction step also converts the 3,6-disubstituted D-mannose residue and the 4-substituted 2-amino-2-deoxy-D-glucose residue into the corresponding alditols-*1-d* (Table II). The remaining 4-substituted 2-deoxy-2-trifluoroacetamido-1-*N*-trifluoroacetyl-β-D-glucopyranosylamine residues should be converted into 4-substituted 2-amino-2-deoxy-D-glucitol-*1-d* residues by analogy with model experiments[14] on D-glucopyranosylamine penta-acetate with 50:1 TFAA/TFA and reduction with sodium borodeuteride. Methylation analysis (Table II) shows that the chains attached by *N*-glycosyl links are intact, with the modifications of the reducing end discussed above.

TFAA/TFA also cleaved the carbohydrate chains attached by *O*-glycosyl links, yielding fraction II (Fig. 1). Removal of the *N*-trifluoroacetyl groups followed by re-*N*-acetylation yielded a product that gave two partly resolved peaks on Sephadex G-25. Sugar analysis of IIR (Table I) and analysis of permethylated IIR showed that


1. TFAA / TFA
2. 50% Acetic acid
3. NaBD$_4$
4. N-Acetylation
D-Galp
D-GlcNAcp
D-Manp
D-GlcNAcp → Asn
D-GlcNAc-ol-1-d
D-Man-ol-1-d

Scheme 1



Scheme 2

it consisted of D-Gal-ol-*1-d* (4%) and β-D-Gal*p*-(1→3)-D-GalNAc-ol-*1-d* (40%). No attempt was made to purify further this fraction. The cleavage of the linkage between the 3-substituted 2-acetamido-2-deoxy-D-galactose residue and serine (or threonine) probably involves acid-catalyzed elimination[15] (Scheme 2) followed by trifluoroacetylation of the C-1 hydroxyl-group formed. The reducing 2-acetamido-2-deoxy-D-galactose residue is further partially degraded[13], generating pertrifluoroacetylated D-galactose. The degradation and reconstitution of the carbohydrate chains attached by *O*-glycosyl links in asialofetuin are shown in Scheme 2.

The present study shows that the carbohydrate chains of asialoglycoproteins can be cleaved by 50:1 TFAA/TFA. The carbohydrate chains attached by *N*-glycosyl links are cleaved by transamidation of the β-D-GlcNAc-(1→*N*)-Asn linkage, and the carbohydrate chains attached by *O*-glycosyl links are cleaved probably by acid-catalyzed elimination from the serine (or threonine) residues. The carbohydrate chains obtained are partly degraded at the reducing end. Since the protein part is degraded by transamidation into products of low molecular weight, the carbohydrate chains can be isolated in a reasonably pure state by gel filtration.

MATERIALS AND METHODS

General methods. — Evaporation was performed under diminished pressure at bath temperatures not exceeding 40°. G.l.c. was performed with a Perkin–Elmer 3920 gas chromatograph equipped with a flame-ionization detector. Separations were performed on (*a*) OV-101 W.C.O.T. glass-capillary columns (25 m × 0.25 mm) at 200° (for partially methylated alditol acetates), (*b*) SE-30 W.C.O.T. glass-capillary columns (25 m × 0.25 mm) at 230° (for partially methylated 2-acetamido-2-deoxy-alditol acetates) or 180–330° (for permethylated oligosaccharide alditols), and (*c*) glass columns (2 m × 0.5 cm) packed with 3% of ECNSS-M on Chromosorb Q at 200° (for alditol acetates). G.l.c.–m.s. was performed on a Varian MAT 311 A instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionization current of 3 mA and an ion-source temperature of 120°. The spectra were processed by an on-line computer system (Spectrosystem 100, Varian MAT).

Analytical methods. — Colorimetric methods were used for the determination of total hexose[16], total hexosamine[17], and sialic acid[18]. Sugar analysis was performed by g.l.c.[19] and m.s.[20] after hydrolysis with 90% aqueous formic acid at 100° for 5 h followed by hydrolysis with 0.25M sulphuric acid at 100° for 18 h. Methylation analysis was performed as previously described[21].

Asialofetuin. — Asialofetuin was prepared from fetuin by mild hydrolysis with acid as previously described[9]. The preparation contained less than 1% of sialic acid.

Degradation of asialofetuin. — A solution of TFAA/TFA (50 ml; 50:1, v/v) was added to freeze-dried asialofetuin (1.0 g) in a sealed glass vessel (250 ml). The reaction mixture was heated at 100° for 48 h (Caution: corrosive mixture under pressure), to give a clear, dark solution that was cooled and evaporated to dryness. The product was dissolved in methanol (25 ml), the solution evaporated to dryness, dissolved in 50% aqueous acetic acid (25 ml) and re-evaporated to dryness, and the residue partitioned between ethyl ether (25 ml) and water (25 ml). The ethereal layer was extracted twice with water (25 ml), and the combined aqueous extracts were washed with ethyl ether (25 ml) and concentrated to 10 ml (1-octanol was added to prevent foaming). This solution was fractionated on a column (3 × 140 cm) of Sephadex G-25 (fine) by elution with 0.1M ammonium acetate buffer (pH 7.0). The separation was monitored by analysis of total hexose, hexosamine, and absorption at 280 nm (Fig. 1). Fraction I (see Fig. 1; 200 mg) was dissolved in water (20 ml) and reduced for 5 h at room temperature with sodium borodeuteride (100 mg). The mixture was acidified with glacial acetic acid and evaporated to dryness. Boric acid was removed from the residue by three co-distillations with methanol (10 ml), and the resulting product was desalted on a column (3 × 140 cm) of Sephadex G-25 by elution with distilled water. The product (170 mg) was dissolved in formamide (10 ml), and a 1:1 mixture of pyridine and acetic anhydride (10 ml) was added. After 20 h at room temperature, ethanol (10 ml) was added to the mixture, and volatile reagents were then removed by evaporation under reduced pressure. The resulting formamide solution was fractionated on a column (3 × 50 cm) of Sephadex LH-20 by elution with acetone–chloroform (2:1; flow rate, 5 ml/min). The acetylated carbohydrates (250 mg) were eluted with the void volume, free from formamide, and were *O*-deacetylated with M ammonia in 75% aqueous methanol (50 ml) at room temperature for 18 h. The mixture was then evaporated to dryness, and the residue was fractionated on a column (2.5 × 80 cm) of Sephadex G-50 by elution with distilled water, to yield reduced and *N*-acetylated fraction I.

Fraction II (see Fig. 1) was reduced with sodium borodeuteride (50 mg) in water (10 ml) for 18 h. The mixture was then acidified with glacial acetic acid and evaporated to dryness. Boric acid was removed from the residue by co-distillations with methanol (3 × 10 ml). The reduced fraction II was acetylated with pyridine (5 ml) and acetic anhydride (5 ml) at 100° for 30 min. Excess of acetic anhydride was decomposed with ethanol (5 ml), and the resulting solution was evaporated to dryness. The residue was partitioned between water (25 ml) and chloroform (25 ml), and the chloroform layer was washed with water (3 × 25 ml) and evaporated to dryness. The acetylated product was *O*-deacetylated with M ammonia in 75% aqueous ethanol (10 ml) at room temperature for 18 h. The solution was then evaporated to dryness, and the residue was fractionated on a column (2 × 45 cm) of Sephadex G-25, to yield reduced and *N*-acetylated fraction II.

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